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Novel Improved Method for Detection of *Dientamoeba fragilis* and Other Parasites.

Background

Dientamoeba fragilis is one of the most common parasites affecting mankind. It is thought to be spread via pinworms acquired by the faecal/oral route and resides in the gastrointestinal tract of the host clinically causing such symptoms as abdominal discomfort, loose motions, bloating, diarrhoea, at times nausea, pruritus ani, malaise and other non specific symptoms. It is perhaps one of the most common parasites residing in the gastrointestinal tract of individuals in the western world and yet few physicians are aware of its presence and its contribution to disease, chiefly due to the fact that it is not diagnosed frequently.

Dientamoeba fragilis (*D. fragilis*) is notoriously difficult to diagnose unless suitable fixatives and permanent staining methods are employed and adequately trained personnel are available (Windsor and Johnson, 1999). Culture methods have been shown to be more sensitive than microscopy at times (Ockert 1990, Sawangjaroen, 1993), however these are not currently used in diagnostic laboratories because of the complexity of the culture methods. However, provided that the methodology is simplified culture could be easier to perform and has the added advantage that the isolates can be lysed and typed, thus aiding future epidemiological studies on top of simple diagnostic studies. *D. fragilis* does not have a resistant cyst stage and consequently cannot survive outside the human host for longer than approximately 12 hours (Sawangjaroen, 1993). In order for the culture method to be successful the culture medium has to be simple and needs to be one that will support the growth of *D. fragilis* and preferably other parasites, eg *Blastocystis hominis*. Furthermore, the medium has to have the features of long shelf-life and transportability. In addition, the detection of the growing parasites needs to be carried out easily by technicians with minimal training. Previous methods have included specific stains, e.g. trichrome and iron-haematoxylin, and more recently Riordan has (US Patent No 5,334,509) suggested an acridine orange or acridine yellow stain for more specifically detecting *D. fragilis*. However, this method unfortunately lacks specificity as it merely stains up RNA/DNA and therefore stains numerous parasites, including non-pathogenic ones. Using this method *D. fragilis* is at times indistinguishable microscopically from such parasites, and so the diagnosis again depends on the availability of highly trained microscopists to diagnose *D. fragilis*. The present invention overcomes this difficulty.

Description of Invention

In this invention the culture method has been simplified to a less complex medium and at the same time improved to one that will support the growth of *D. fragilis, Blastocystis hominis* (*B. hominis*), and other parasites including other amoebae (for example E. *histolytica / dispar*

being another pathogenic one). This novel medium (which also doubles as a transport medium) uses an egg slope as opposed to previously used saline agar, and demonstrates enhanced reliability of culture. The medium is no longer complex as those described in the past comprising of numerous chemicals. The current medium is - by culture standards - greatly simplified yet more reliable. Unlike the use of live E.coli bacteria (not suitable to give to patients to collect specimens), this novel medium has been designed to work even more reliably without E.coli. A further advance is in the formulation of the liquid phase which has added horse serum and Bactopeptone(1-40%, but typically 20%). In addition a novel container system has been designed to allow the faecal sample to be placed immediately onto the culture medium by the patient. Then, the culture medium will be transported to the laboratory for incubation while *D. fragilis* and other parasites survive the transportation due to the unique nature of the medium which doubles as a transport medium. This novel container, a specimen bag containing the faecal container, also has the culture medium, a sachet and a small scoop. Only a 'pea-sized' amount of stool is required and it is placed onto the culture medium with the contents of the enclosed sachet added to make the transport/culture simplified. See *Figure 1*.

The culture medium is bi-phasic and consists of a solid phase (egg or simple agar slope) in a liquid face. The liquid formulation per 100mls includes typically 90mls Phosphate Buffered Saline pH 7.4 (range 6.8 – 7.8), 5mls of Sterile Horse Serum(1-15mls) and 5mls 20% Bactopeptone(1-15mls). Five drops of 0.5% erythromycin(may be 0.1-2.0%) are added to the culture medium and a small amount of rice starch is also added in the form of the sachet. Rice starch is essential for the xenic cultivation of intestinal protozoa (Clark and Diamond, 2002). Once the specimen is received in the laboratory it is incubated at 37°C for 24 hours. Then an extra 2 drops of erythromycin are added together with a small amount of rice starch, and a further incubation is carried out for 24 hours (48 hours incubation in total) before microscopic examination is carried out. Further examinations may be carried out at 3 and finally at 4 days to allow for the occasional detection of slow-growing parasites. A drop of sediment is then examined using the X20 objective of the microscope for the typical morphology of *Dientamoeba*. *D. fragilis* ingests the rice starch voraciously, differentiating it from *B. hominis* when viewed under the microscope. Under microscopic observation *D. fragilis* appear as round, refractile bodies packed with rice starch. Other intestinal amoebas such as *Entamoeba* and *Iodamoeba* also ingest rice starch, but *D. fragilis* produces characteristic pseudopodia after 10-20 minutes at room temperature. These pseudopodia are leaf-like and are easily distinguishable from those produced by *Entamoeba*. Positive cultures can be simply confirmed by making a smear of the deposit, allowing it to air dry and fixing it

in industrial methylated spirit or ethanol. This then can be stained with Giemsa (10% in PBS pH6.8) for 20 minutes with a wash of buffer before examining under the microscope.

Other parasites and mixed infections may also be detected using the faecal culture method. Any parasite growing in the culture can be identified further by using the simple stain. *Entamoeba* sp grow much larger than *D. fragilis* and the pseudopodia are much more obvious and larger. Any query re: E. *histolytica/dispar* isolates can be resolved by lysing using 0.25ml 0.25% SDS containing 0.1 M EDTA and sending for a specific PCR/ELISA to confirm / exclude the pathogenic *E. histolytica*. Similarly, *B. hominis* can be detected using this culture. Although the pathogenicity of this parasite is controversial it has been associated with IBS. It is possible that a certain subtype of *B. hominis* may be linked with disease, again a lysate can be made and then typed using riboprinting.

Clinical Examples

Example 1.

In a 34y old female suffering with longstanding loose motions, wind and mild bloating a clinical diagnosis of 'Irritable Bowel Syndrome' was made. To exclude enteric parasitic infestation a stool test was ordered by the patient's physician. A faecal sample was collected by the patient using a small scoop provided. A pea-sized amount of stool was placed into the novel culture medium, which in this case contained the erythromycin already. The contents of the sachet containing the rice starch was also added. The specimen was taken to the laboratory where it was incubated for 24 hours at 37°C. A small amount of rice starch was later added as well as 2 drops of erythromycin and the culture incubated a further 24 hours. A drop of the sediment was transferred onto a glass slide and a coverslip added. This preparation was examined under a light microscope using the x20 objective. Round, refractile bodies that had ingested rice starch granules and produced delicate leaf-like pseudopodia after 10-20 minutes at room temperature were identified and a presumptive diagnosis of *D. fragilis* was made. A smear was made, allowed to air-dry, fixed in IMS and was simply stained with Giemsa for confirmation. The patient was treated with the appropriate anti-parasitic therapy and recovered.

Example 2.

In a 49y old male patient with a family history of bowel malignancy complaining of marked flatulence and hepatic-flexure cramping pain a colonoscopy was carried out to exclude the presence of bowel cancer. Simultaneously a sample of luminal contents was collected by aspiration. The colonscopy-acquired material was collected into the specialized faecal container and a small portion was transferred into the culture medium. The contents of the sachet containing the rice starch was also added to the medium. The specimen was transported to the laboratory where it was incubated for 24 hours at 37°C. A further small amount of rice starch was now added together with 2 drops of erythromycin and the culture was incubated for a further 24 hours. A drop of the sediment was transferred onto a glass slide and a cover slip added. This preparation was examined under a light microscope using the x20 objective. Round, refractile bodies that ingest rice starch granules and produce delicate leaf-like pseudopodia after 10-20 minutes at room temperature were detected and a presumptive diagnosis of *D. fragilis* was made. A smear was made, allowed to air-dry, fixed in IMS and simply stained with Giemsa for confirmation.

Example 3

A female patient 39y of age presented with long standing gastrointestinal symptoms including abdominal pain, flatulence, distention, nausea and minimal weight loss. Repeated faecal samples were negative at the local hospital microbiology laboratory. In the most recent stool sample refractile bodies were observed using direct microscopy, although these could not be identified and did not show up in the parasite concentration method. [The laboratory concerned did not use permanent faecal stains e.g. trichrome or iron-haematoxylin. This is a common feature in Australia and the UK, where very few routine laboratories employ such methodologies]. Part of the specimen was transferred into the novel culture medium, using the small scoop provided within the kit. The contents of the sachet containing the rice starch was also added to the medium. The specimen was transported to the laboratory where it was incubated for 24 hours at 37°C. A further small amount of rice starch was now added together with 2 drops of erythromycin and the culture was incubated for a further 24 hours. A drop of the sediment was transferred onto a glass slide and a cover slip added. This preparation was then examined under a light microscope using the x20 objective. Microscopic analysis showed numerous refractile bodies, some of which ingested the rice starch. The Giemsa stain demonstrated the presence of both *D. fragilis* and *B. hominis*, a common finding in patients presenting with IBS-like symptoms.

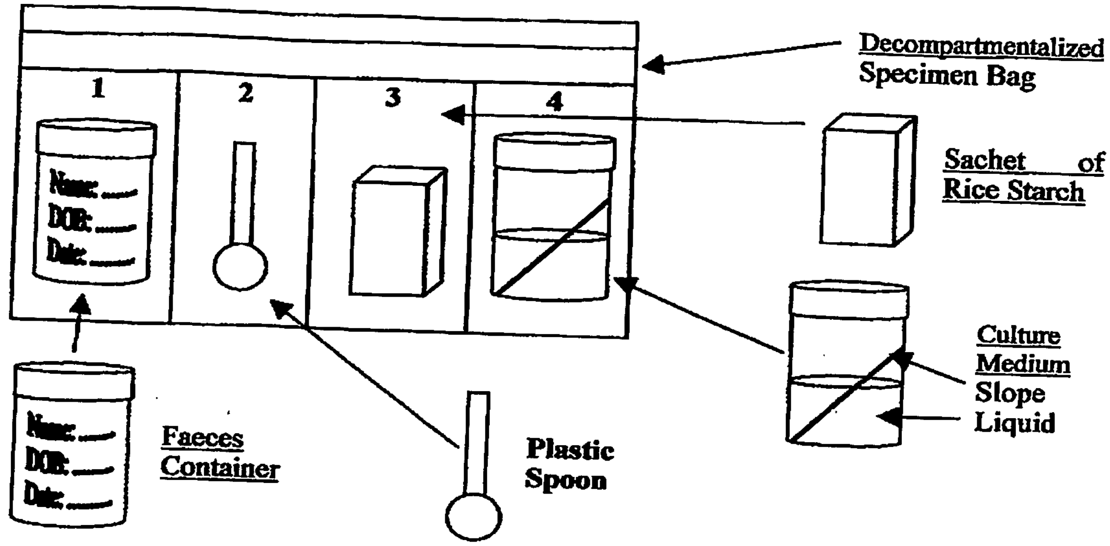

Figure 1

- Collect faeces into faeces Container (1)
- Inoculate a small amount of faeces into culture medium (4), using a plastic spoon.
- Discard plastic spoon into compartament 2 and seal. This can be discarded in the laboratory.
- Add sachet of rice starch (3) to culture medium (4).
- Send whole pack to laboratory.
- The feaces container can be used for C & S and other parasites (OVA).
- The culture medium is incubated.

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